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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : <b>G01N 21/64</b>	<b>A1</b>	(11) International Publication Number: <b>WO 00/62042</b>
		(43) International Publication Date: 19 October 2000 (19.10.00)

(21) International Application Number: PCT/EP00/03185

(22) International Filing Date: 7 April 2000 (07.04.00)

(30) Priority Data:  
9901306-2 9 April 1999 (09.04.99) SE

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB,  
BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM,  
DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,  
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,  
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,  
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ,  
UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH,  
GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR,  
IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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*With international search report.*

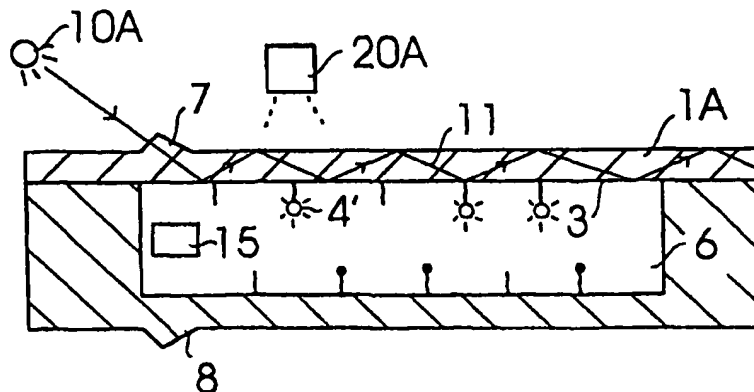
*Before the expiration of the time limit for amending the  
claims and to be republished in the event of the receipt of  
amendments.*

(54) Title: SAMPLE CUVETTE FOR MEASUREMENTS OF TOTAL INTERNAL REFLECTION FLUORESCENCE

(57) Abstract

A biochemical sample chamber (6) is made of a transparent material. At least two of the inner chamber walls (2, 3) act as substrates and are provided with catchers (5), such as chemical or affinity reagents. In use, the catchers are coupled to fluorescent groups (4) within the sample in a pattern representative for the sample. The fluorescent groups (4) of each chamber wall (2, 3) are excited, using total internal reflection technique, by light introduced to prisms (7, 8) corresponding to each inner

wall which has been provided with catchers. The excited fluorescent groups emit light that is detected. Thus, the inner space of the chamber is used with high efficiency, thereby enabling an analyzing capacity which is least twice that of a single chamber.



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## SAMPLE CUVETTE FOR MEASUREMENTS OF TOTAL INTERNAL REFLECTION FLUORESCENCE

**TECHNICAL FIELD OF THE INVENTION**

- 5 The present invention relates to equipment for so-called total internal reflection fluorescence analysis (TIRF), and more specifically to a chamber for accommodating a sample for TIRF analysis.

**PRIOR ART**

- 10 Total internal reflection fluorescence (TIRF) is an optical technique utilized in measurement of the presence of fluorescent molecules at solid-liquid interfaces, such as the interface between a transparent substrate, e.g. a silica substrate, and a liquid, e.g. water, exhibiting a lower index of refraction than the substrate.

15

TIRF is based on the principle that a beam of light that is traveling through the substrate is totally reflected against the interface and back into the substrate provided that the beam of light is travelling with a proper angle of incidence. Thus, the substrate acts as a waveguide. The basic principle for

20 TIRF measurement utilized with the present invention is, for example, described in US 5,512,492, US 5,667,166, WO 9427137 and WO 9735203, all of which are incorporated by reference. TIRF substrates, each having a wall being provided with patches of different catcher compositions are described in WO 9427137 and WO 9735203, respectively.

25

- The incident beams and the reflected beams interfere in the substrate to produce an electromagnetic standing wave at the interface. The standing wave yields an exponentially decaying electric field, the evanescent field, on the outside of the substrate. The evanescent field decays over a distance of
- 30 approximately 1000 to 2000 Å.

- The TIRF technology utilizing planar waveguides and measurement of fluorescence from the waveguide surface has earlier been used for assaying the presence or absence of analytes in samples. The analytes have typically
- 35 been organic compounds (biomolecules) having one or more structures

selected among, for instance, peptide structures (including oligo- and/or polypeptide structures), carbohydrate structures, nucleotide/deoxynucleotide structures (including nucleic acid structures), lipid structures (including steroid and glyceride structures) etc. This use has included qualitative as well as quantitative measurements. See Herron et al., SPIE Advances in Fluorescence Sensing Technology Vol 1885 (1993) 2-8 and 28-39, and Tony Murray, Resequencing Genes Using Arrayed Primer Extension (APEX), presented at international Business Communications 3rd Annual Conference on BIOCHIP ARRAYS and integrated devices for clinical diagnosis, 5-6 March 1997, at Sheraton San Diego Hotel and Marina, San Diego, CA.

Regardless of the method used, conventional TIRF analysis equipment is characterized in that a chamber is provided for accommodating a sample to be analyzed, and "catchers" are attached to the inner surface of one wall of the chamber.

The term "catcher" is used throughout the present application as a general term to indicate a chemical reactant or certain kinds of affinity reactants coupled directly or via a linker, for instance a polymer or the like, to a substrate surface. The reactant is a chemical compound that can be associated with a corresponding chemical compound such that there is a mutual attraction between the compounds of the pair. Thus, examples of chemical ligands include the individual members of the thiol/reactive disulphide pair and the epoxy/amine pair. Affinity reactants include the individual members of the lectin/carbohydrate pair, the nucleic acid/complementary nucleic acid pair, the enzyme/substrate pair, the enzyme/cofactor pair and the biotin/streptavidin pair. Particularly important pairs of affinity reactants in the context of the present invention are those amongst which at least one, preferably both, of the members in a pair doesn't exhibit a polypeptide structure. This latter in particular applies to pairs of complementary nucleic acids.

Typically, the sample is added to the chamber together with suitable reagents, a suitable buffer solution and fluorescent groups, such as fluorescently labeled nucleotides. After a dwell time, during which the fluorescent groups

are bound to the catchers, the remaining sample is washed away and substituted by an aqueous solution. Light is introduced into the chamber wall provided with the catchers, and the resulting fluorescent light is registered for further steps of analysis.

5

There are technical limitations to the obtainable catcher surface density. Therefore, in order to extract as much information as possible from the same sample a sample is often transferred from one chamber, which is provided with a first set of catchers and wherein the sample is analyzed in a first step,  
10 to a second chamber, which is provided with a second set of catchers and wherein the sample is analyzed in a second step. Actually, even more steps, including more chambers, may be utilized.

However, there is a continuous need for improved analyzing capacity of such  
15 equipment.

#### **SUMMARY OF THE INVENTION**

The present invention seeks to provide TIRF equipment and methods with improved analyzing capacity.

20

This object is achieved with a device in accordance with claim 1, a system in accordance with claim 7 and a method in accordance with claim 9.

According to the present invention, it is possible to perform more than one  
25 analyzing steps using one chamber only, thereby avoiding the time consuming transfer of the sample from one chamber to another chamber in sequence. This is achieved by providing the chamber with numerous activated chamber surfaces for detecting fluorescent groups.

30 Further features, advantages and applications of the present invention will become apparent from the detailed description given hereinafter.

#### **BRIEF DESCRIPTION OF DRAWINGS**

The present invention will become more fully understood from the detailed description given below and the accompanying drawings, which are given by way of illustration only, and wherein:

- 5 Fig. 1 is a schematical perspective view of a sample chamber for use with the present invention;

Fig. 2 is a cross sectional view of the chamber of Fig. 1, taken along line I-I and illustrating catchers disposed according to an embodiment of the  
10 present invention;

Fig. 3 is a cross sectional view according to fig. 2, illustrating a first analyzing step according to the present invention;

- 15 Fig. 4 is a cross sectional view according to fig. 2, illustrating a second analyzing step according to the present invention;

Fig. 5 is a schematical perspective view of another embodiment of a sample chamber for use with the present invention;

20

Fig. 6 is a schematical perspective view of a set of sample chambers according to the present invention disposed in a rotating disk;

- Fig. 7 is a schematical perspective view of the rotating disk of fig. 6,  
25 illustrating the fluorescence detection arrangement;

Fig. 8 is a cross sectional view similar to the view of Fig. 3, showing an alternative embodiment of light introducing prisms;

- 30 Fig. 9 is a cross sectional view similar to the view of Fig. 3, showing yet another embodiment of a light introducing means

#### **DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

A first embodiment of a chamber according to the invention is shown  
35 schematically in the perspective view of Fig. 1, and in cross-section of Fig. 2.

The chamber 6 is formed in a cell 1, said cell being made of any suitable transparent material, such as glass or a chemically resistant plastic. The cell is typically assembled from an upper part 1A sealed with a suitable adhesive to a lower part 1B which is provided with recesses forming the chamber 6, an  
5 inlet channel 14 and an outlet channel 15, as is well known in the art.

The upper cell wall is provided with a prism 7 for introducing a ray of light at an angle with respect to the upper cell wall into the upper wall. Similarly, the lower cell wall is provided with a prism 8 for introducing a ray of light at an  
10 angle with respect to the lower cell wall into the lower wall.

Thus, the chamber 6 formed inside the cell 1 exhibits an upper inner wall 3 and a lower inner wall 2. Using any conventional method suitable for the purpose, such as the earlier mentioned method according to US patent  
15 5,807,522, catchers 5 are attached to these upper and lower inner walls 2, 3.

Depending on the method used, fluorescent groups 4 are provided on the catchers 5, either as separate components in a sample containing DNA and/or RNA, or as groups that in a previous step have already been coupled  
20 to the DNA or RNA groups of the sample. Although not new in itself, an illustrative, not limiting example of this shall be described in some detail.

The protocol for the provision of fluorescent groups on each inner chamber surface 2,3 provided with suitable catchers, as described above, includes the  
25 steps of

- (i) introducing a liquid into the chamber 6 via the inlet channel 14, said liquid including fluorescent groups that are capable of becoming bound to the catchers on the inner chamber surface 2,3, and
- (ii) incubating for a sufficient period of time for the fluorescence groups to  
30 become bound, if possible, to the catcher.

The duration of the incubation step will differ depending on the reagents used. When very fast reactions are utilized, the incubation duration may approach zero or close thereto, meaning that those two steps in principle may  
35 coincide.

The fluorescent group may be a fluorescence-doped substance selected from the group consisting of individual members of the above-mentioned pairs of affinity or chemical reactants, with preference for nucleic acids (DNA, RNA, nucleic acid analogues such as LNA, PNA etc), and epoxy- or amino-containing compounds, thiol- or reactive disulfide-containing compounds etc.

The catcher on the inner walls 2,3 of the chamber may be located in a number of sub-areas, for instance in the form of spots, on each respective inner wall. The catcher of each sub-area may differ with respect to kind and/or density (number of molecules per unit area). The number of sub-areas (spots) per unit area (cm<sup>2</sup>) may be from two up to 100, 1000, 10.000 or even 100.000. Appropriate techniques for applying different kind of catchers to small sub-areas are known in the field, as is suggested in US 5.445.934 and US 5.807.522, hereby incorporated by reference.

After preparation, as described with the steps i) and ii) above, the inner walls of the chamber are prepared for analysis in that those catchers 5 that where sensitive to components present in the liquid are now provided with fluorescent groups 4.

According to the invention, following the preparation steps the chamber walls that have been provided with catchers are analyzed in sequence. More specifically, in a first analyzing step one of the chamber walls is irradiated with light using the TIRF principle to energize the fluorescent groups attached to that wall, followed by the detection of these groups with a fluorescence detecting device. In a second analyzing step the measures of the first step are repeated for a second inner wall. Thus, the analyzing steps are repeated for each wall of the chamber that has been provided with catchers.

The analyzing steps are typically run with the chamber filled with the appropriate liquid, preferably an aqueous solution.

In certain embodiments, a general cleaning step for restoring the chamber could be performed after each analyzing step.



The first and second analyzing steps described above are illustrated for a double-sided TIRF chamber in Fig. 3 and 4, respectively.

5 In the first analyzing step, a substantially monochromatic light source 10A, such as a laser or a lamp with a filter for selecting the proper wavelength, emits light that is guided into the upper part 1A of the cell through a prism 7. The prism 7 and the position of the light source 10A are selected such that the ray of light 11 hits the interface between the upper wall 3 and the liquid in  
10 the chamber 6 at the angle of incidence necessary to produce a total internal reflection. The ray of light propagates in the wall, thereby producing an evanescent field that excites the fluorescent groups 4' associated with that wall. These groups 4' now emit light. A fluorescence detecting device 20A, such as a CCD camera with a suitable filter, is arranged to monitor the  
15 transparent upper wall area of the upper cell part 1A to detect and register those positions where excited fluorescent groups emit light for further analysis in a subsequent step not described herein.

In one embodiment of the present invention the second analyzing step, which  
20 is performed after the first analyzing step, corresponds to the first analyzing step in substantially all respects, i.e. the incoming ray of light 12 from a second source of light 10B enters through a prism 8 in the lower part 1B of the cell to excite the fluorescent groups 4 of the lower wall 2 catchers 5. A second fluorescence-detecting device 20B registers the positions for the  
25 present fluorescent groups.

A second embodiment of the invention differs from the embodiment described above in that only one source of light 10A and one fluorescence-detecting device 20A are provided, making Fig. 3 useful also for this second  
30 embodiment. The prisms 7, 8 are disposed symmetrically with respect to each other. Thus, the second analyzing step of the method of the invention is performed by simply turning the cell 1 around to position the previously lower cell wall upwards. Of course, the same effect is achieved by changing the positions of the light source 10A and the fluorescence-detecting device 20A

instead of turning the cell, or by redirecting the light with any suitable reflecting device.

Actually, provided that the cell walls and the liquid are transparent enough,  
5 the fluorescence-detecting device 20A could be provided at one side only of the chamber, regardless if one or several light sources are used. By correlating the readings from the fluorescence-detecting device 20A with information on which wall is actually irradiated, the fluorescence-detecting device 20A is able to register the fluorescent groups through the opposing wall  
10 of the chamber.

When designing a chamber according to the invention, care should be taken to ensure that opposing walls for attaching fluorescent groups are not disposed too close to each other. If the opposing walls are separated a  
15 distance less than about 2000 Å there is a risk that the evanescent field generated when a first wall of the chamber is irradiated with light will affect also the fluorescent groups of a second opposing wall. If, in such a case, the fluorescent groups on the second wall are also excited and emit light, these will interfere with the readings of the light emitting groups of the first wall.

20 It should be noted that any of the prisms could, alternatively, be positioned inside the wall, as is illustrated with the prisms 107 and 108 in the detail view of Fig. 8. In Fig. 8, which shows a ray of light 111 from a light source 110 being introduced via the prism 107, all the components, except for the prisms,  
25 are similar to their corresponding components of Fig. 1, 2 and 3.

Even other alternative means for introducing the light are useful. For example, the light introducing means described in US patent 5,512,492 is useful with the invention of the present invention. In Fig. 9 is shown an  
30 embodiment wherein a ray of light 211 from a light source 210 is introduced through the end surface 207 of an upper cell plate 201A covering the chamber 206. A bottom cell plate 201B, having an end surface 208 adapted for introducing light, forms the bottom of the chamber 206. For the rest, the components of the embodiment according to Fig. 9 are similar to the  
35 corresponding components of the embodiment shown in Fig. 1, 2 and 3.

An embodiment of a chamber 31 according to the invention is shown in Fig. 5. The chamber 31 is provided with four inner walls 32-35, each inner wall being provided with catchers (shown for two inner walls hidden in Fig. 5). Prisms 38A-D are provided at one end of each outside wall, respectively. The end openings 36, 37 act as inlet and outlet channels. For simplicity, light sources as well as fluorescence-detecting devices are not shown.

As is obvious, the method described above is useful also for the chamber 31 of Fig 5, wherein the analyzing step, according to the invention, is repeated for each wall, i.e. four times.

It is also obvious that the number and positions of walls, light sources and fluorescence-detecting devices could be altered in any useful way, while still being within the scope of the invention.

The method and the device of the invention provide the advantage that a number of analyzing steps, corresponding to the activated wall surfaces within the chamber, could be performed without transferring the sample to different chambers.

Thus, the time for analyzing is shortened since the steps of incubation and washing need not to be repeated for each chamber.

Furthermore, each step of transferring the sample gives rise to losses, since some components of the sample always diffuses into the transferring liquid, and is wasted during washing. The present invention avoids this.

Still further, space is saved using the invention since a chamber according to the invention provides more analyzing area per unit of spatial area than a conventional single-side chamber does.

Fig. 6 and 7 shows an embodiment of the invention wherein a number of double-sided chambers (three are shown in Fig. 6, one of which is shown in Fig. 7) are disposed in a disc member 42.

The disc 42 is made from a double layer transparent plastic material. Recesses are formed in opposing surfaces of the layers to form chambers 41, inlet channels 43 and outlet channels 44 when the layers are properly  
5 sandwiched to form the disc 42, as is well known within the art.

Depending on the application at hand, the inlet channels 44 and the outlet channel 43 of each chamber 41 are in fluid communication with any suitable means, such as a pressure building device, another chamber, a sample inlet  
10 port, a waste outlet etc. (not shown).

The upper side of the disc 42 is provided with prisms 45, one for each chamber, while the lower side of the disc 42 is provided with corresponding prisms 46 (not shown for clarity), one for each chamber 41.  
15

The disc member 42 is adapted to be rotated around its axis. For this reason, the disc 42 is provided with a central hole 47 for accommodating a rotating shaft of a driving device (not shown).

20 In use, the chambers 41 are filled with sample liquid simultaneously or in any selected order. The analysis is made according to the steps of the invention described earlier.

By rotating the disc 42 around its center it is possible to analyze numerous  
25 chambers in a simple way using only one light source 51 and one fluorescence-detecting device 53 to detect excited fluorescent groups 4". Of course, the use of multiple light sources and fluorescence-detecting devices improves the productivity even further.

30 Thus, using the embodiment according to Fig. 6 and 7, a very compact high-productivity multi-chamber analyzing station is provided. According to the invention, the inner space of the chamber is used with high efficiency. It is possible to at least double the analyzing capacity of a single chamber.

A chamber according to the invention has numerous uses. A non-limiting example of this is the use for assaying an analyte in a sample, as indicated in the introductory part of the present application. This use comprises the steps:

5 (a) incubating the inner walls of the substrate chamber of the present invention with a liquid containing a fluorescent group and possibly an analyte; and,

(b) detecting by TIRF the occurrence of the fluorescent group on each wall separately.

10 The conditions for step (a) are selected to provide binding of the fluorescent group to the inner walls via a catcher on each respective wall in a manner which reflects the presence/absence and/or the amount of the analyte in the liquid. See, for example, the publications on TIRF referred to in the introductory part of the present application. In the most important variants  
15 this means that a complex comprising the fluorescent group and the catcher will be formed on at least one of the inner walls, when the analyte is present in the liquid. The level of total internal reflection fluorescence (TIRF) of the fluorescent group of the surface will therefore in these variants represent the presence/absence and/or amount of the complex for a certain combination of  
20 catcher, fluorescent group and analyte, and thus also the presence/absence and/or amount of the analyte concerned with the combination in the liquid incubated in step (a). In certain variants the fluorescent group and the analyte as present in the liquid may be the same entity, i.e. the analyte is fluorescent, for instance a fluorescently labeled analyte analogue.

25

During step (b), the fluorescent group bound to the inner wall will respond to the evanescent field created by the TIRF method to emit light that is detectable with any suitable equipment, thereby providing information about the presence/absence and/or amount of the analyte as present in the liquid  
30 introduced into the chamber.

Between steps (a) and (b) there may be one or more steps in which the liquid used in step (a) is replaced with liquids not containing compounds disturbing the measurement of the light emitted by the fluorescent group bound to the  
35 inner wall, for instance washing liquids.

By using two or more fluorescent groups and/or two or more different catchers it will be possible to assay for two or more analytes simultaneously. In this case each wall may have different sets of catchers, for instance with  
5 different kind and/or density of catchers in separate sub areas of each inner wall.

The term analyte in the context of this example of use includes an original analyte as it occurs in an original sample or a processed original analyte  
10 (analyte analogue) as it appears in a processed sample. An analyte analogue is related to the presence/absence and/or amount of the analyte in the original sample. Depending on the analyte, sample, assay protocol etc. the original sample with its original analyte or a processed sample together with an original analyte or an analyte analogue will or will not be included in the  
15 liquid placed in the chamber in step (a).

It is obvious that the present invention, as illustrated with the embodiments described above, may be varied in many ways, for example by combining features of different embodiments. Such variations are not to be regarded as a  
20 departure from the scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

**CLAIMS**

1. A chamber (6, 31, 41), for performing total internal reflection fluorescence (TIRF) measurement, provided with at least two walls (2, 3; 32-35) and  
5 characterized in that at least two of the walls (2, 3; 32-35) at selected areas are provided with catchers (5).
2. The chamber (6, 31, 41) of claim 1,  
10 characterized in that the chamber (6, 31, 41) includes a pair of diametrically opposed walls (2, 3; 32, 34), both said walls at selected areas being provided with catchers (5).
3. The chamber (31) of claim 2,  
15 characterized in that the chamber (31) further comprises a second pair of diametrically opposed walls (33, 35), said second pair of walls being essentially perpendicularly disposed with respect to said first pair of walls (32, 34), and at least one of the walls of said second pair being provided with catchers (5).  
20
4. The chamber (41) of claim 2,  
characterized in that said chamber is integrally formed within a circular disc adapted to rotate around its axis.
- 25 5. The chamber (6, 31, 41) according to anyone of the claims 1 through 4,  
characterized in that the catchers (5) are chemical reactants selected from the group consisting of the individual members of the thiol/reactive disulphide pair and the epoxy/amine pair.  
30
6. The chamber (6, 31, 41) according to anyone of the claims 1 through 4,  
characterized in that the catchers (5) are affinity reactants selected from the group consisting of the individual members of affinity pairs in which at least  
35 one, preferably both, of the members doesn't comprise polypeptide structure.

7. A fluorescence detection system (10A, 10B, 20A, 20B, 6; 51, 53, 41) for use with a sample chamber according to any of the previous claims, characterized in that it comprises
- 5 means (10A, 10B; 51) for light irradiation of each chamber wall being provided with catchers having fluorescent groups attached thereto; and means (20A, 20B; 53) for detecting light emitted from the fluorescent groups on each such chamber wall.
- 10 8. The fluorescent detection system of claim 7, characterized in that the light irradiating means (10A, 10B; 51) is sequentially operative on each respective chamber wall being provided with catchers, and the means (20A, 20B; 53) for light detection is operative on each chamber surface subsequently to its irradiation by the irradiating means.
- 15 9. A method for analyzing a biochemical sample comprising the steps of:
- filling a chamber with a sample, said chamber being provided with at least two inner surfaces each being provided with catchers for interaction with
  - 20 components in the sample;
  - introducing fluorescent groups that may or may not be capable of becoming bound to the catchers on the inner chamber surface (2,3);
  - incubating for a sufficient period of time for fluorescence groups to become bound to the catcher;
  - 25 - irradiating one of the chamber walls being provided with catchers with light using the total internal reflection technique to energize the fluorescent groups attached to that wall;
  - detecting the energized fluorescent groups with a fluorescence detecting device; and
  - 30 - repeating the steps of irradiation and detection for each chamber wall being provided with catchers.

10. The method according to claim 9,



characterized in that the catchers of the inner chamber surfaces are selected among the group of chemical reactants consisting of the individual members of the thiol/reactive disulphide pair and the epoxy/amine pair.

- 5 11. The method according to claim 9,  
characterized in that the catchers of the inner chamber surfaces are selected  
among the group of affinity reactants consisting of the individual members of  
affinity pairs in which at least one, preferably both, of the members doesn't  
comprise polypeptide structure.

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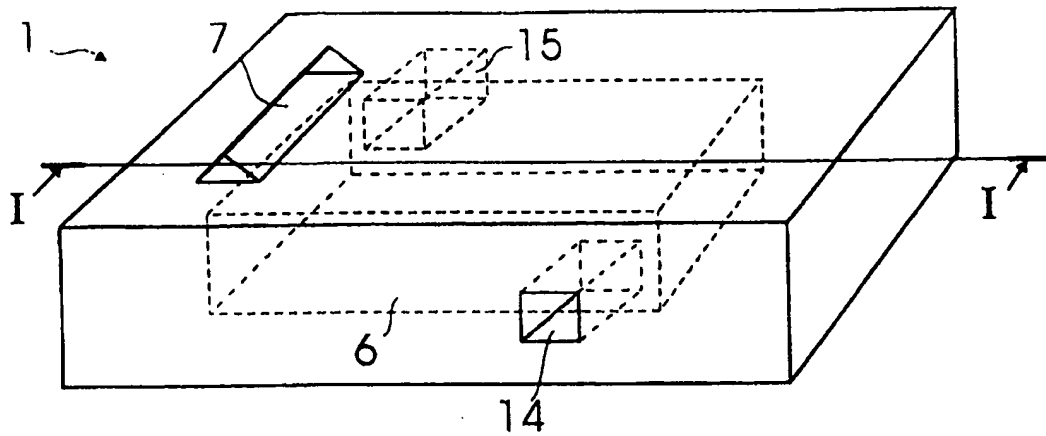


Fig. 1

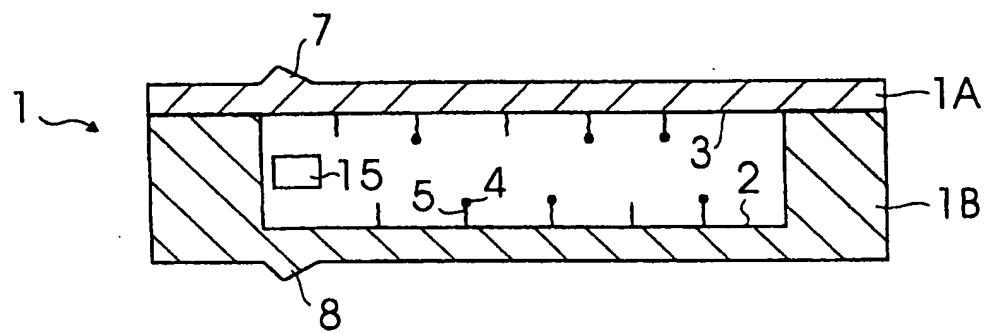


Fig. 2

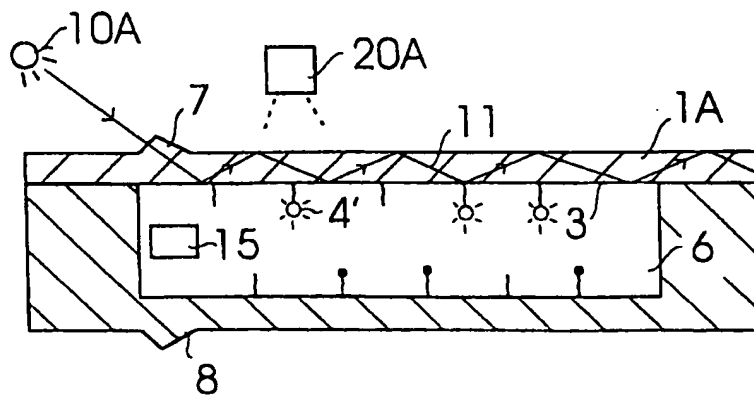


Fig. 3

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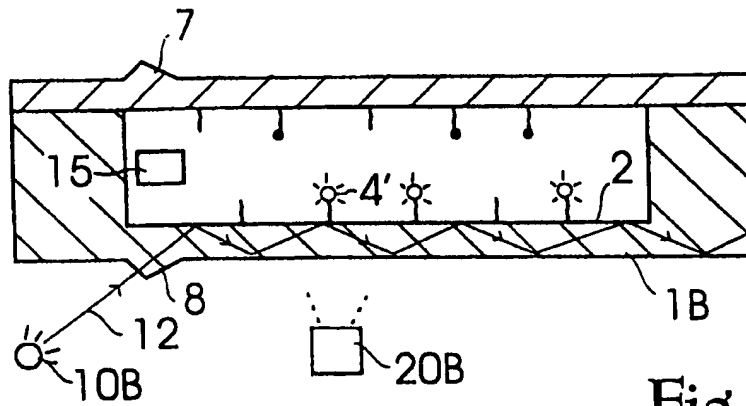


Fig. 4

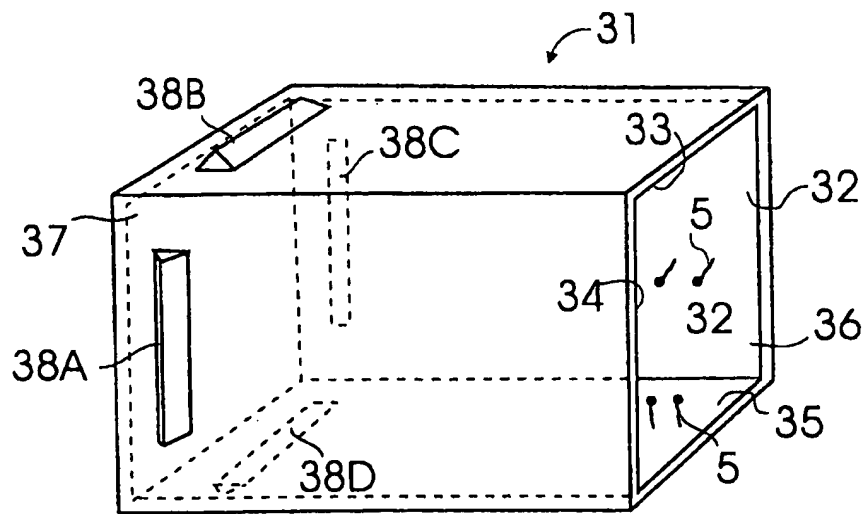


Fig. 5

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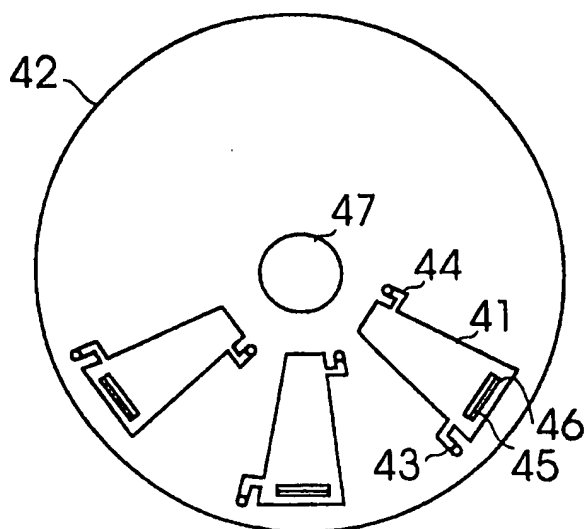


Fig. 6

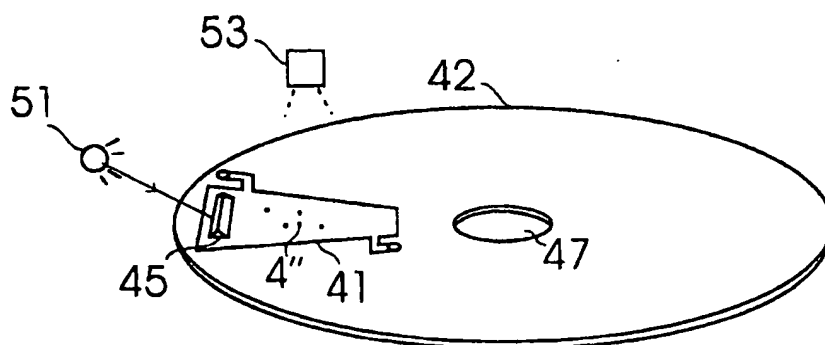


Fig. 7

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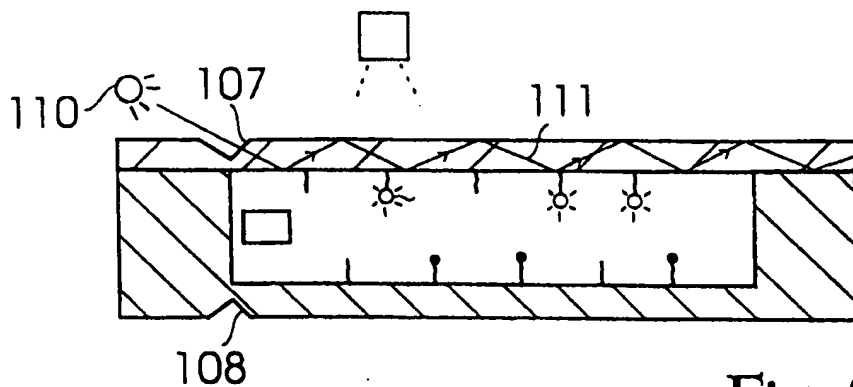


Fig. 8

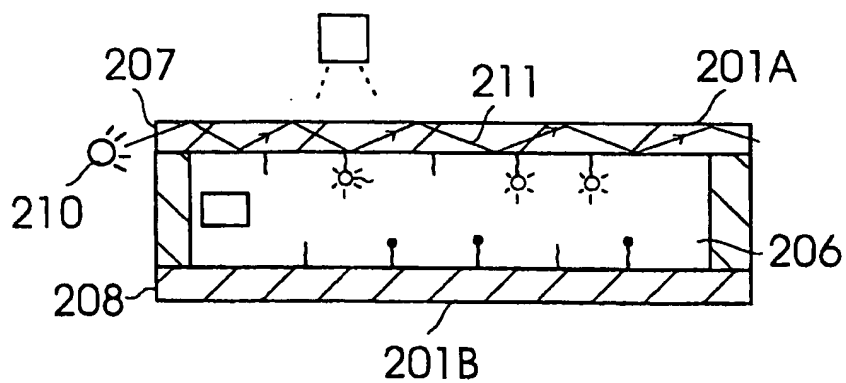


Fig. 9

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/03185

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, INSPEC, COMPENDEX, BIOSIS, IBM-TDB

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 184 600 A (BATTELLE) 18 June 1986 (1986-06-18) page 1, line 4 -page 2, line 6 page 4, line 16 - line 34 page 14, line 18 -page 15, last line page 16, line 22 -page 17, line 8	1,2,7-9
Y	claims 1,2,4-6; figures 4,5,9; example 6	4,5,10
Y	WO 96 35940 A (CIBA-GEIGY) 14 November 1996 (1996-11-14) page 1, paragraph 1 - paragraph 4 page 13, last paragraph -page 14, line 23 page 25, line 18 page 25, line 24 -page 26, line 1 figures 3,4	4
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

4 August 2000

Date of mailing of the international search report

14/08/2000

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# INTERNATIONAL SEARCH REPORT

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PCT/EP 00/03185

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	<p>EP 0 664 451 A (ABBOTT LABORATORIES)  26 July 1995 (1995-07-26)  page 3, line 45 -page 4, line 2  page 5, line 33 - line 51  page 6, line 24 - line 44  page 6, last paragraph -page 7, line 16  -----</p>	5,10

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